

FORM PTO-1390 (REV 10-94)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371				9374.21USWO
				U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5) Unknown 09/763791
INTERNATIONAL APPLICATION NO. PCT/GB99/02845	INTERNATIONAL FILING DATE August 27, 1999	PRIORITY DATE CLAIMED August 28, 1998		
TITLE OF INVENTION INGESTIBLE COMPOSITIONS COMPRISING ANTIBACTERIAL AGENTS				
APPLICANT(S) FOR DO/EO/US BLAKE et al.				
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:				
<p>1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.</p> <p>2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371</p> <p>3. <input checked="" type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(I).</p> <p>4. <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.</p> <p>5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2))</p> <p>a. <input checked="" type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau).</p> <p>b. <input checked="" type="checkbox"/> has been transmitted by the International Bureau.</p> <p>c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US)</p> <p>6. <input type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)).</p> <p>7. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))</p> <p>a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau).</p> <p>b. <input type="checkbox"/> have been transmitted by the International Bureau.</p> <p>c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired</p> <p>d. <input checked="" type="checkbox"/> have not been made and will not be made.</p> <p>8. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).</p> <p>9. <input checked="" type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).</p> <p>10. <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).</p> <p>Items 11. to 16. below concern document(s) or information included:</p> <p>11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.</p> <p>12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.</p> <p>13. <input checked="" type="checkbox"/> A FIRST preliminary amendment. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment.</p> <p>14. <input type="checkbox"/> A substitute specification.</p> <p>15. <input type="checkbox"/> A change of power of attorney and/or address letter.</p> <p>16. <input checked="" type="checkbox"/> Other items or information: International Search Report; International Preliminary Search Report; Demand; Request</p>				

U.S. APPLICATION NO (If known, see 37 C.F.R. 1.5) Unknown 09/763791		INTERNATIONAL APPLICATION NO PCT/GB99/02845		ATTORNEY'S DOCKET NUMBER 9374.21USWO	
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17. <input checked="" type="checkbox"/> The following fees are submitted: BASIC NATIONAL FEE (37 CFR 1.492(a) (1)-(5)): Search Report has been prepared by the EPO or JPO.....\$860.00 International preliminary examination fee paid to USPTO (37 CFR 1.492(a)(1)).....\$690.00 No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)).....\$710.00 Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(3)) paid to USPTO \$1000.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4)\$100.00				CALCULATIONS PTO USE ONLY	

ENTER APPROPRIATE BASIC FEE AMOUNT =				\$860.00	
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				\$0	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	37	-20 = 17	X \$18.00	\$306.00	
Independent claims	11	-3 = 8	X \$80.00	\$640.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ \$260.00	\$0	
TOTAL OF ABOVE CALCULATIONS =				\$1806.00	
Reduction by 1/2 for filing by small entity, if applicable. Small entity status is claimed pursuant to 37 CFR 1.27				\$0	
SUBTOTAL =				\$1806.00	
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				+ \$0	
TOTAL NATIONAL FEE =				\$1806.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property				+ \$0	
TOTAL FEES ENCLOSED =				\$1806.00	

		Amount to be: refunded	\$0
		charged	\$0

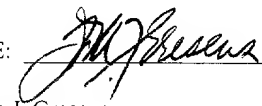
a. ☒ Check(s) in the amount of \$1806.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of \$ _____ to cover the above fees
A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 13-2725.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO
 John J. Gresens
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 Minneapolis, MN 55402-0903

SIGNATURE: 
 NAME: John J. Gresens
 REGISTRATION NUMBER. 33,112

S/N unknown

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	BLAKE et al.	Docket No.:	9374.21USWO
Serial No.:	unknown	Filed:	concurrent herewith
Int'l Appln No.:	PCT/GB99/02845	Int'l Filing Date:	August 27, 1999
Title:	INGESTIBLE COMPOSITIONS COMPRISING ANTIBACTERIAL AGENTS		

CERTIFICATE UNDER 37 CFR 1.10

'Express Mail' mailing label number: EL658341455US

Date of Deposit: February 26, 2001

I hereby certify that this correspondence is being deposited with the United States Postal Service 'Express Mail Post Office To Addressee' service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

By: 
Name: Brian Maharaj

PRELIMINARY AMENDMENT

Box PCT
Assistant Commissioner for Patents
Washington, D. C. 20231

Dear Sir:

In connection with the above-identified application filed herewith, please enter the following preliminary amendment, based on claims amended in prosecution of the international application and published in the International Preliminary Examination Report, a copy of which is enclosed herewith:

IN THE SPECIFICATION

A courtesy copy of the present specification is enclosed herewith. However, the World Intellectual Property Office (WIPO) copy should be relied upon if it is already in the U.S. Patent Office.

IN THE CLAIMS

Please amend the following claims (marked-up copy attached):

3. (Amended) A formula feed according to claim 1, in which the concentration of XOR exceeds the normal physiological concentration of XOR.
4. (Amended) A formula feed according to claim 1, which includes up to 500µg/ml active xanthine oxidoreductase.
6. (Amended) A formula feed according to claim 1, in which the formulation includes buttermilk, the buttermilk including active XOR.
7. (Amended) A formula feed according to claim 1, in which the formulation is in liquid form.
8. (Amended) A formula feed according to claim 1, the formulation further including one or more electron donors.
9. (Amended) A formula feed according to claim 1, the formulation further including one or more electron acceptors.
10. (Amended) A formula feed according to claim 1, which is suitable for feeding a human infant.
11. (Amended) A combination product for use in the preparation of a formula feed according to claim 1, in which the product comprises two separate portions, the first portion including active XOR and the second portion being sterilized and comprising substantially no active XOR.
13. (Amended) A combination product according to claim 11, in which the second portion has been heat treated.

14. (Amended) A combination product according to claim 11, in which the first portion has been pasteurized.

15. (Amended) A combination product according to claim 11, in which the first portion is in a first container, and the second portion is in a second container.

18. (Amended) A composition according to claim 16, the composition being in the form of a powder.

20. (Amended) A method according to claim 19, a composition for use in the treatment of gastrointestinal infection, the composition being suitable for addition to a formula feed, and the composition comprising active XOR in combination with one or more electron donors and/or one or more electron acceptors.

21. (Amended) A method according to claim 19, the method comprising the steps of:

- a. preparing a first portion of the formula feed, comprising a composition including active XOR; and
- b. preparing a second portion of the formula feed, the first portion and second portion being separate from each other for subsequent mixing to form the formula feed.

24. (Amended) A method according to claim 21, in which the first portion has been subjected to a heat treatment step in which its temperature does not exceed 65° C.

25. (Amended) A method according to claim 21, in which the second portion comprises a sterilized feed composition.

Please add the following new claims:

36. (New) A method according to claim 19, a composition comprising of buttermilk.

37. (New) A method according to claim 19, a composition being in the form of powder.

REMARKS

The above preliminary amendment is made to remove multiple dependencies from claims 3, 4, 6, 7, 8, 9, 10, 11, 13, 14, 15, 18, 20, 21, 24 and 25.


Applicants respectfully request that the preliminary amendment described herein be entered into the record prior to calculation of the filing fee and prior to examination and consideration of the above-identified application.

If a telephone conference would be helpful in resolving any issues concerning this communication, please contact Applicants' primary attorney-of record, John J. Gresens (Reg. No. 33,112), at (612) 371.5265.

Respectfully submitted,

MERCHANT & GOULD P.C.
P.O. Box 2903
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Dated: February 26, 2001

By  _____
John J. Gresens
Reg. No. 33,112

JJG/tvm

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3. (Amended) A formula feed according to claim 1 [or claim 2], in which the concentration of XOR exceeds the normal physiological concentration of XOR.
4. (Amended) A formula feed according to [any one of claims 1 to 3] claim 1, which included up to 500 µg/ml active xanthine oxidoreductase.
6. (Amended) A formula feed according to [any one of claims 1 to 5] claim 1, in which the formulation includes buttermilk, the buttermilk including active XOR.
7. (Amended) A formula feed according to [any one of claims 1 to 6] claim 1, in which the formulation is in liquid form.
8. (Amended) A formula feed according to [any one of claims 1 to 7] claim 1, the formulation further including one or more electron donors.
9. (Amended) A formula feed according to [any one of claims 1 to 8] claim 1, the formulation further including one or more electron acceptors.
10. (Amended) A formula feed according to [any one of claims 1 to 9] claim 1, which is suitable for feeding a human infant.
11. (Amended) A combination product for use in the preparation of a formula feed according to [any one of claims 1 to 10] claim 1, in which the product comprises two separate portions, the first portion including active XOR and the second portion being sterilized and comprising substantially no active XOR.
13. (Amended) A combination product according to claim 11 [or claim 12], in which the second portion has been heat treated.

14. (Amended) A combination product according to [any one of claims 11 to 13] claim 11, in which the first portion has been pasteurized.

15. (Amended) A combination product according to [any one of claims 11 to 14] claim 11, in which the first portion is in a first container, and the second portion is in a second container.

18. (Amended) A composition according to claim 16 [or claim 17], the composition being in the form of a powder.

20. (Amended) A method according to claim 19, [the composition being according to any one of claims 16 to 18] a composition for use in the treatment of gastrointestinal infection, the composition being suitable for addition to a formula feed, and the composition comprising active XOR in combination with one or more electron donors and/or one or more electron acceptors.

21. (Amended) A method according to claim 19 [or claim 20], the method comprising the steps of:

- a. preparing a first portion of the formula feed, comprising a composition including active XOR;
- and
- b. preparing a second portion of the formula feed, the first portion and second portion being separate from each other for subsequent mixing to form the formula feed.

24. (Amended) A method according to [any one of claims 21 to 23] claim 21, in which the first portion has been subjected to a heat treatment step in which its temperature does not exceed 65° C.

25. (Amended) A method according to claim 21 [or claim 22], in which the second portion comprises a sterilized feed composition.

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Ingestible compositions comprising
antibacterial agents

5 The present invention relates to compositions comprising xanthine oxidoreductase (XOR). In particular, but not exclusively, the invention relates to the use of a composition comprising XOR as a feed for humans or animals, and finds special application in relation to
10 formula feed for babies.

 The enzyme xanthine oxidoreductase (XOR) is a complex molybdoflavoprotein, the action of which has been studied for many years.

 XOR is a major protein component of the membrane
15 surrounding fat droplets in whole milk. Consequently, cows' milk is a rich and convenient source of the enzyme, as has been known for many years. XOR has also been characterised from rat, chicken and turkey livers. More recently, human milk has been found to contain XOR, which
20 has now been purified from that source. Initial research shows that, while the human milk XOR enzyme has similar physicochemical properties to the bovine milk enzyme, the human enzyme shows differences in its catalytic activity.

 The purpose of XOR in milk has never been fully
25 ascertained. One suggestion has been that it provides a source of molybdenum and iron, metals potentially useful for the developing neonate.

 There have also been suggestions that XOR has a role in the production of bactericidal agents. As
30 discussed further below, XOR can catalyse the production of superoxide and hydrogen peroxide, which are known bactericidal agents. As indicated below, however, in the low oxygen concentrations found in the gut, it is thought

unlikely that bactericidal levels of superoxide or hydrogen peroxide will be attained.

The role of XOR in the development of gout has been the subject of extensive study. XOR is involved in the catabolism of purines to uric acid, catalysing the oxidation of hypoxanthine to xanthine and xanthine to uric acid. Compositions have been developed which block the action of XOR and which are useful in the prevention and treatment of gout.

XOR exists in two inter-convertible forms, xanthine dehydrogenase (XDH, EC 1.1.1.204) and xanthine oxidase (XO, EC 1.1.3.22). XDH, which is believed to predominate *in vivo*, preferentially reduces NAD^+ , whereas XO does not reduce NAD^+ , preferring molecular oxygen. Where reference is made herein to xanthine oxidoreductase, it should be understood that that term refers to both xanthine dehydrogenase (XDH) and xanthine oxidase (XO), where appropriate. It will be appreciated that references to XOR further include references to analogs of xanthine oxidoreductase that have xanthine oxidoreductase activity. Such analogs may include but are not limited to, for example, xanthine oxidoreductase which has been modified chemically or otherwise, analogs having fragments of xanthine oxidoreductase derived from naturally-occurring enzyme, and analogs having polypeptides obtained by replication of the enzyme or a portion thereof using any suitable biotechnological method, provided in each case that the catalytic activity of the endogenous xanthine oxidoreductase is retained at least to an appreciable extent.

XOR can also effect reduction of molecular oxygen. That reaction generates the reactive oxygen species superoxide anion and hydrogen peroxide.

The superoxide radicals thus generated have been implicated in relation to chronic inflammatory intestinal diseases such as Crohn's disease and colitis ulcerosa.

US 5,484,605 describes the administration of oxypurinol
5 to the intestine to inhibit xanthine oxidase, thus preventing the formation of superoxide radicals which are believed to act as inflammatory mediators.

It has been reported, Millar, T.M. et al, FEBS Letters 427 (1998) 225-228, that, under hypoxic
10 conditions and in the presence of NADH, XOR is capable of catalysing the reduction of glyceryl trinitrate (GTN), as well as inorganic nitrate and nitrite, to nitric oxide (NO).

Nitric oxide (NO) is widely recognised as
15 mediating the relaxation of smooth muscle in vasodilation and as initiating many other important biological functions, including inhibition of platelet aggregation and adhesion. Its generally accepted physiological source is NO synthase, a complex enzyme which is totally
20 dependent on oxygen for its activity and consequently ineffective in a hypoxic environment, where the vasodilatory properties of NO might be seen to be advantageous.

Babies who are not breast-fed are fed what is
25 referred to herein as formula feed. Such formula feed has a composition which commonly includes sources of protein, fat and carbohydrate as well as minerals and are generally formulated to be nutritionally complete. Many formula feeds are based on cow's milk while some others
30 are soy based. The term "formula feed" used herein should be understood to cover both formulations based on milk products as well as those based on soya or other products and which may or may not be nutritionally

complete. The formula feed commonly takes the form of a dried powder which is reconstituted before being fed to the baby. Alternatively, the formula feed may be in liquid form either as a concentrated liquid requiring
5 dilution or as a ready-to-feed formulation.

Enteral feeding may be prescribed where a patient is unable to eat normally, or has severe malabsorption or malnourishment. Enteral feed may take the form of tube and sip feeds. The enteral feed may be nutritionally
10 complete and generally comprises protein, carbohydrate and fat as well as vitamins and minerals. The term "enteral feed" used herein should be understood to cover both formulations based on milk products as well as those based on soya or other products and which may or may not
15 be nutritionally complete. The enteral feed may be in liquid form or may be in the form of a powder which is reconstituted before use. In many cases the enteral feed formulation is based on the composition of the neonatal formula feed.

20 The present invention provides a formulation for use as a bactericidal agent in the human or animal digestive system, the formulation including active xanthine oxidoreductase.

More particularly, in accordance with the
25 invention, there is provided a formula feed formulation or enteral feed formulation for administration to a human or animal, the formulation including active xanthine oxidoreductase (XOR).

The term "feed" is used herein to include both
30 enteral feeds and formula feeds.

We have found that the active enzyme xanthine oxidoreductase (XOR) is absent from formula feeds and from enteral feeds. It is thought that that is because

XOR is either absent from the feed or it is inactivated as a result of the treatment process used in the production of feeds. Where reference is made herein to "active" XOR and "active" enzyme, it is to be understood that reference is made to XOR and enzyme which has not, for example, been inactivated or broken down in such a way.

Up to now, it would have been thought that the addition of active XOR to feeds would be unnecessary and undesirable. The only roles of XOR previously known, as indicated above, were as a general "housekeeping" enzyme in the catabolism of purines, which may also have a pathological role in the development of gout and of chronic inflammatory intestinal diseases. The only other beneficial role of XOR was thought to be as a source of molybdenum and iron. It was thought (correctly) that those minerals could still be obtained from inactivated XOR, which might be present in feed.

We have now found that the addition of active XOR to feed is potentially beneficial in killing pathogenic intestinal bacteria. The active XOR may thus be regarded as a "natural antibiotic", that is, a substance of natural origin which is capable of destroying or inhibiting the growth of at least some strains of pathogenic micro-organism. It is believed that feed containing active XOR will be beneficial in the mitigation of intestinal infection and necrotising enterocolitis in the neonate fed with formula feed. Sick adults that are enterally fed are also at risk of the same complications as children fed formula feed.

Studies have shown that babies who are fed with formula feed are about twenty times more likely to suffer from gastrointestinal (GI) infection than babies who are

breast fed. The reason for that discrepancy has not been known.

In the presence of oxygen, as stated above, XOR can generate superoxide and hydrogen peroxide. However, the amount of oxygen available in the gut is generally very low and bactericidal levels of superoxides or hydrogen peroxide are unlikely to be attained.

As discussed above, it has been found that the catalysis by XOR of the reduction of glyceryl trinitrate (GTN), as well as inorganic nitrate and nitrite, to nitric oxide (NO) occurs under hypoxic conditions. It is such hypoxic conditions which are present in the gut.

We have also found that the optimum pH for the production of NO under the hypoxic conditions is about pH 5.5. It is known that the pH of the neonatal stomach normally ranges between pH 3.5 and 6. Thus, we have found that the neonatal stomach presents an environment in which the production of NO in the presence of XOR is at a peak. Furthermore, it is at pH levels above about 4 at which pathogenic bacteria are more active than at lower pH of about 2 normally found in the adult stomach. Thus it is believed that the neonatal stomach, having a relatively high pH, is at particular risk from pathogenic bacteria. Furthermore, it is found that, for example in post-operative adults, the pH of the stomach rises to above 4. It has also been noted that post-operative adults have a relatively high risk of GI infection.

Thus, surprisingly, we have found under the conditions of the neonatal gut, XOR can catalyse the production not only of superoxide, but of NO. Superoxide and NO rapidly interact to generate peroxynitrite, a much more potent bactericidal species than superoxide, NO or hydrogen peroxide. While superoxide has some

bactericidal properties and in some situations NO has also been found to kill or damage bacteria, it is the interaction of superoxide and NO to form peroxynitrite and other products which is believed to give superior bactericidal action. Peroxynitrite (and, it is thought, other products of the interaction of superoxide and NO) are particularly potent bactericidal species.

The concentration of nitrite present in the neonatal gut is normally low, and it might therefore be thought that the potential for XOR-catalysed generation of NO would be limited. It is believed however that, the known affinity of XOR for acidic polysaccharides such as those occurring in bacterial capsules causes XOR to become more concentrated in the immediate vicinity of bacteria. In anaerobic environments, bacteria commonly are found to excrete nitrite and thus the association of XOR with the bacteria may have the result that the XOR will be located in a localised region of elevated nitrite concentration.

In one embodiment of the invention, the formulation is for use as formula feed. As indicated above, the use of XOR finds particular application in relation to the feeding of neonates.

Alternatively, the formulation is for use as an enteral feed for adults.

While reference is made herein to the feeding of humans, the invention is also of particular relevance in the feeding of animals and the terms "formula feed", "enteral feed" and "feed" should be understood to include formulations for animals. The invention finds particular application in respect of mammals.

Animals also suffer from GI infection, in particular Scours disease (diarrhoea in neonatal animals). Scours disease is a particular problem for calves and pigs taken

from their mothers soon after birth. For calves and pigs taken from their mothers up to ten days after birth and fed waste milk or formula feed, the mortality rate can be as much as 80%. In some cases, Scours disease can be
5 cured by administering electrolyte solutions but such treatment is very expensive.

It is thought that the addition of active XOR to feed for animals will mitigate GI infections, in particular Scours disease.

10 The formulation may advantageously include an amount of active XOR which is such that the active XOR concentration in the formulation is at least as large as the normal physiological concentration of XOR, for example in milk. Preferably, the concentration of the XOR
15 exceeds the normal physiological concentration of XOR. Advantageously, the formulation includes from 50 to 500 μ g/ml of XOR, based on the volume of the formulation (when ready-to-use, having been diluted if necessary). Preferably the formulation includes from 50 to 150 μ g/ml
20 which is comparable to the level of XOR found in natural breast milk. In some cases, it may be desirable to increase or reduce the amount of active XOR in the feed, for example, as a function of the baby's age and/or the incidence of GI infection.

25 We have found that a particularly advantageous source of active XOR is buttermilk, in particular lyophilised buttermilk.

In some cases it is thought that the addition of XOR in purified or other form will be desirable, for example
30 where there is a risk of allergy to buttermilk.

The formula feed and/or the enteral feed including the active XOR in accordance with the invention may be in

the form of a powder or may be in liquid form ready for feeding to the patient.

As indicated above, it is believed that for the formula feed and enteral feed formulations, any active XOR which was naturally present in those formulations is destroyed or deactivated during the manufacture of the formulation, possibly as a result of heat treatment. In a particularly preferred embodiment of the invention, one portion of the formula or enteral feed is prepared in the standard way, for example including heat treatment step(s). A composition comprising active XOR is subsequently added to the prepared portion. That addition of the XOR may be carried out by a manufacturer, or may be carried out, for example, immediately prior to use of the formulation. For example, in the case of a powdered feed which is made up with water prior to use, a powdered composition comprising the active XOR may be contained in a separate container from that of the rest of the formulation, for example in a sachet.

Alternatively, the portion including the active XOR may be in tablet form or may be contained in a capsule. The rest of the feed may be made up in the normal way, for example by the addition of hot water and shaking, and the XOR composition added once the rest of the feed has been prepared. That is of particular importance in the case in which the normal method of making up of the formulation might deactivate the XOR, for example as a result of the addition of boiling water. Where the making up of the formulation would not damage the activity of the XOR, or where the formulation is sold ready-prepared, the XOR composition and the rest of the formulation may be provided together as a mixture.

Thus the invention provides a combination product for use in the preparation of a formulation in which the product comprises two separate portions, the first portion including active XOR and the second portion comprising substantially no active XOR.

In one embodiment of the invention, the second portion of the formulation is in the form of a powder.

As indicated above, the second portion may have been treated by heat treatment, for example UHT treatment, in the normal way, thus deactivating the XOR.

In a particularly preferred embodiment of the invention, the active XOR is added in the form of buttermilk. The first portion may be pasteurised. As indicated above, pasteurisation has been found not to deactivate XOR.

As indicated above, advantageously, the first portion is in a first container, the second portion is in a second container. Thus the two portions can be held separate until use. For example, where both portions are in the form of a powder, each powder portion can be made up separately using different methods before being mixed together. For example, the first portion might be made up with cold water and the second portion may be made up with boiling water. Alternatively, the first portion may be in the form of a liquid ready for mixing into the second portion of the formulation once the second portion has been made up.

Advantageously, the formulation further includes electron donors and/or electron acceptors. Examples of electron donors are purines and nitrogen-containing heterocyclic compounds, for example hypoxanthine, NADH. Examples of electron acceptors are organic and inorganic nitrates and nitrites.

According to the invention, there is also provided a composition for addition to a formulation for use as feed, the composition comprising active XOR. It is envisaged that the composition containing the active XOR might be sold separately from the formula or enteral feed, the composition being for addition to the feed prior to use.

Advantageously, the composition comprises buttermilk. As indicated above, buttermilk is a particularly preferred source of active XOR. Lyophilised buttermilk may be used. It may be preferable, however, for the buttermilk to be spray-dried by spraying the buttermilk into air at a temperature which is so selected that the activity of the XOR is wholly or at least substantially retained. In general, to avoid any detrimental effect on the activity of XOR, heat treatment steps that may be used in the treatment of XOR-containing compositions or formulations should preferably be such that the temperature of the composition or formulation does not exceed 65°C.

The composition may be in the form of a powder.

Advantageously, the composition further includes electron donors and/or electron acceptors. Examples of electron donors are purines and nitrogen-containing heterocyclic compounds, for example hypoxanthine, NADH. The nature of the electron donor may influence the rate of generation of bactericidal species. For example, use of hypoxanthine as electron donor results in a more rapid, but less prolonged generation of bactericidal species than NADH. Examples of electron acceptors are organic and inorganic nitrates and nitrites.

According to the invention, there is also provided, a method of making a formulation for use as feed, the

method comprising the step of adding a composition comprising active XOR.

The method advantageously comprises the steps of:

- a. preparing a first portion of the formulation, the first portion comprising a composition including active XOR; and
- b. preparing a second portion of the formulation, the first portion and second portion being separate from each other for subsequent mixing to form the formulation.

Advantageously, the first portion comprises buttermilk, preferably lyophilised buttermilk. As indicated above, the buttermilk may include additives.

Preferably, the second portion comprises a sterilised feed composition.

Also provided by the invention is the use of active XOR in the treatment of gastrointestinal (GI) infection. The invention also provides the use of active XOR in the treatment of Scours disease.

Additionally provided is the use of active XOR in the killing of bacteria.

The invention also provides a method of feeding a patient with enteral feed, in which the enteral feed includes active XOR and also a method of feeding a neonate with formula feed, in which the formula feed includes active XOR.

Also provided is a method of treatment of gastrointestinal infection using active XOR.

It will be appreciated that XOR used in accordance with the invention may be of any biological origin, for example of mammalian or other animal origin, or originating from a suitable micro-organism, for example,

aspergillus sp. XOR of ruminant origin offers the advantage of ready availability.

The invention will now be explained in more detail with reference to the accompanying drawings, of which:

5 Fig. 1 is a graph illustrating the effect of peroxynitrite on cell viability, under the conditions of Test 8(a);

10 Fig. 2 is a graph illustrating the effect of added peroxynitrite on cell colony growth in semi-skimmed-bovine milk, as determined in Test 8(b);

Fig. 3 is a graph illustrating the dependence of XO-mediated killing of cells as determined in Test 8(c);

15 Fig. 4 is a graph showing the effects of hypoxanthine addition on bacterial growth in pasteurised milk;

Fig. 5 is a graph showing the effect of XO concentration on bacterial growth rate;

Fig. 6 is a graph showing the dependence of cell growth inhibition on hypoxanthine concentration;

20 Fig. 7 is a graph showing the effect of oxypurinol on XO/hypoxanthine-related growth inhibition; and

Fig. 8 is a graph showing the effect of hypoxanthine at various concentrations on growth inhibition.

25

Determination of nitric oxide production

The production of nitric oxide in the following tests and examples was analysed using an ozone chemiluminescence assay in a continuous flow apparatus
30 (Sievers NOA 280) that allows the real time analysis of NO production. The apparatus was modified to allow a constant stream of nitrogen to flow into the reaction chamber. Chemiluminescence data were collected by a data

acquisition system; the mean NO produced in parts per billion (ppb) was calculated from readings taken every second and shown as ppb or mV.

Progress curves, of molar production of NO against
5 time, were calculated by taking into account the gas flow and the mean level of NO. Molar production of NO was expressed as ppb/sec or mV/sec. Reactions were carried out in a final volume of 1 ml at 37°C in an atmosphere of < 1% oxygen (Stathkelvin combination needle oxygen
10 electrode, Diamond General Corp.).

The method used was as follows:

(a) Two clean 7ml bijous were obtained, one for each of the "substrates" and the "milk".

To the "substrates" bijou, 200µl of 100mM stock sodium
15 nitrite was added together with 200µl of the 5mM reduced NADH to give an assay concentration of 20mM nitrite and 1mM reduced NADH.

To the "milk" bijou were added 600µl of milk sample to give an assay volume of 1ml.

20 (b) Using a flow rate of 200ml/min, each bijou mixture and a corresponding injection needle was degassed with nitrogen gas (N₂) for about 10 seconds and the bijou was capped.

(c) The reaction cell comprised a 7ml screw-cap
25 bijou having three needle holes in its cap. A continuous flow of warmed N₂ (200ml/min flow rate) was injected into the bijou through one of the needle holes in the cap to give the required hypoxic conditions. The reaction cell was held at a temperature of 37°C in a water bath mounted
30 on a magnetic stirrer. A magnetic flea was placed in the reaction cell to mix the samples once injected.

(d) Samples of NO were taken from the reaction cell by a needle that was connected to a Sievers Nitric Oxide

Analysers (NOA-280) and the results were recorded for analysis using a computer.

(e) At time (t) 0 minutes, the NOA-280 started the measurement of NO from the reaction cell. At t=1 minute, the contents of the "substrates" bijou were injected into the reaction cell using a 1ml syringe and the background NO was measured.

(f) At t=5 minutes, the contents of the "milk" bijou were injected into the reaction cell using a 1ml syringe and the release of NO was monitored for a further 20 minutes. The set up was such that the reaction cell was a sealed system having an inlet gas flow of 200ml/min and a sample extraction flow to the NOA-280 of 200ml/min. The steady-state generation of NO (which corresponded to a plateau region on the trace of the NO production) was noted to give the mV/s release of NO from the 1ml assay volume.

The samples were diluted with PBS where necessary to give an assay of 1ml for the test of NO generation.

Reagents used

The reagents used in the tests and examples described below were as follows:

1. Bovine xanthine oxidase (XOR) - Biozyme, Blaenavon, UK.

This source of enzyme had a concentration of 10.7mg/ml and was batch 104AX

2. Sodium nitrite (NaNO_2) - Sigma Chemicals (Sigma-Aldrich Company Ltd.), Poole, UK. This was dissolved in 1X Phosphate Buffered Saline (PBS), pH 7.3, to the required concentration.

3. β -Nicotinamide Adenine Dinucleotide, reduced form, (β -NADH) - Sigma Chemicals.

4. Phosphate Buffered Saline (PBS) - Oxoid Ltd., Basingstoke, UK. Tablets were added in the proportion of 1 per 100ml of distilled water and mixed until thoroughly dissolved to give 1X PBS, pH7.3.
- 5 5. Oxypurinol - Sigma Chemicals. A stock 1mM solution was made up by adding 0.0015g to 0.25ml of 1M NaOH. This was mixed until the oxypurinol dissolved. Then 9.75ml of 1X PBS was added and the pH altered until pH7.3 was reached using drops of 1M HCl.
- 10 6. Diphenyliodonium (DPI) - ICN Biomedical. A stock 1mM solution was made up by adding 0.0032g to 10ml 1X PBS, pH7.3.
7. Formula milks:
 - 15 a. PreAptimil with Milupan (2.4g protein/100ml) - Milupa, Trowbridge, UK.
 - b. Aptamil First with Milupan (1.5g protein/100ml) - Milupa.
 - c. Farleys First Milk - H.J. Heinz Co. Ltd., Hayes, UK.
 - 20 d. Cow and Gate Premium (1.4g protein/100ml) - Cow and Gate, Trowbridge, UK.
 - e. Sma Gold 1.5g protein/100ml) - SMA Nutrition, Maidenhead, UK.
 - f. Sma Wysoy (1.8g protein/100ml) - SMA Nutrition.
- 25 8. Human Breast Milk. Samples obtained from subjects in the local area
9. Carton milk. Milk bought in pints from the local shop either as full milk or semi-skimmed milk (3.4g protein/100ml). Lordswood Dairy, Bristol, UK was the
- 30 source of the milk.
10. Untreated cows' milk was obtained from a local farm
11. Buttermilk obtained from Waitrose (John Lewis Partnership, UK).

Test 1

The release of nitric oxide (NO) from a composition including pure bovine xanthine oxidase under hypoxic conditions was studied. The samples studied comprised reduced β -Nicotinamide adenine dinucleotide (NADH) and nitrite (NO_2^-) and pure bovine xanthine oxidase.

Bovine xanthine oxidase was diluted with PBS as indicated in Table 1 below to give 300 μ l of enzyme mixture. The enzyme mixture (300 μ l) was mixed with 700 μ l of a substrate mixture to give an assay volume of 1 ml. The substrate mixture comprised nitrite which was added to the assay to give a concentration of 1mM and NADH which was added to give a concentration of 0.3mM. Where, for example in Tests 2 to 4 below, additional components are added to the sample, the amount of PBS is adjusted accordingly to give an assay volume of 1 ml. The total XOR protein in the assay is shown in Table 1. The sample was placed in the reaction vessel of the NO determination apparatus under a nitrogen atmosphere and the generation of NO was measured and the results calculated as a steady state rate in mV/s. The rate of NO release for the different concentrations of XOR is also shown in Table 1.

Table 1

Volume XOR enzyme (μ l)	Volume PBS (μ l)	XOR protein in assay (μ g)	NO release (mV/s)
0	300	0.0	15.10
2	298	21.4	50.05
5	295	53.5	76.35
10	290	107.0	128.55
15	285	160.5	187.20
20	280	214.0	225.75

- 5 Where the pure XOR is used in the samples, NADH was required as a substrate for the reduction of the nitrite to proceed. It is believed that in many cases, where the XOR is added to, for example a formula feed, the addition of a separate electron donor, for example NADH, will not
10 be required because suitable substrate species will already be present in the feed and/or in the GI tract of the infant or adult to whom the feed is administered.

Test 2

- 15 Using the method described above in respect of Test 1, the effect of the variation in the concentration of NADH on the production of NO was investigated. 4mM NADH was diluted with PBS to give the relevant concentration. The results are shown in Table 2.

Table 2

XOR protein in assay (μ g)	NADH concentration in assay (mM)	NO release (mV/s)
53.5	0.00	0.00
53.5	0.10	30.45
53.5	0.25	66.80
53.5	0.50	92.55
53.5	1.00	103.35
53.5	2.00	109.20
107.0	0.00	0.00
107.0	0.10	74.45
107.0	0.25	142.70
107.0	0.50	192.65
107.0	1.00	230.05
107.0	2.00	264.65

5 Test 3

Using the method described above in respect of Test 1, the effect of the variation in the concentration of nitrite on the production of NO was investigated. As for Test 1, the concentration of NADH was 0.3 mM. 1M nitrite (or 100mM nitrite, where appropriate for low concentrations) was diluted with PBS to give the relevant concentration. The results are shown in Table 3.

Table 3

XOR protein in assay (μg)	Nitrite concentration in assay (mM)	NO release (mV/s)
21.4	0	0.00
21.4	1	53.75
21.4	5	184.8
21.4	10	378.10
21.4	25	407.55
21.4	50	474.05

5 Test 4

The method of Test 1 was repeated with known inhibitors of XOR included in the assay to show that the NO generation was being catalysed by XOR. Two different inhibitors were used. Oxypurinol was used at a concentration of 100 μM . Oxypurinol is a molybdenum site-specific inhibitor. Diphenyliodonium (DPI) was used at a concentration of 100 μM . DPI is a FAD site inhibitor. The results are shown in Table 4.

15 Table 4

Inhibitor	NO release (mV/s)
100 μM Oxypurinol	0.00
100 μM DPI	0.00

There was no release of NO in the presence of the XOR inhibitors.

Test 5

Full fat milk was assayed with 20mM nitrite and 1mM NADH as described above in Test 1. The milk had a protein content of 3.4g/100ml. 600µl of milk was used in each test giving a protein content in each assay of 20.4mg.

The milk was taken and divided into two halves. One was kept at 4°C, and the other at -20°C. Samples of the milk were taken over several days and the generation of NO was investigated using the method described above. The results are given below in Table 5.

Table 5

Day	4°C - NO release (mV/s)	-20°C - NO release (mV/s)
1	77.57	73.00
2	75.50	90.05
3	68.67	52.65
6	626.1	73.35
8	Over 1000	39.20

It was found that the milk which was kept at 4°C started to go off after 3 days and that that was accompanied by a large increase in NO release which was not inhibited by 100µM oxypurinol or DPI. Therefore, when the milk goes off it is probable that bacteria in the milk are starting to produce NO from the nitrite. The milk kept frozen retained its activity and did not go off.

Samples of fresh full fat milk assayed with 100µM Oxypurinol or DPI were tested and found to give no

detectable NO generation. Thus full fat milk does include a source of active XOR which produces NO under hypoxic conditions with nitrite and NADH.

5 Test 6

Human breast milk was assayed with 20mM nitrite and 1mM NADH using the method as described above. The human breast milk was collected on several days post partum and frozen at -20°C. 600µl of milk was used in each test.

- 10 The generation of NO was investigated using the method described above. The results are given below in Table 6.

Table 6

Days post partum	XOR protein content (µg/ml)	NO release (mV/s)
7	411.26	40.45
30	683.05	60.75
36	561.49	42.48
66	305.52	27.80
158	110.84	29.80

15

Test 7

A selection of formula feeds (formula milks) were taken and assayed with 20mM nitrite and 1mM NADH as described above. The formula milks were tested for the generation of NO. The Cow and Gate Premium was left for 7 days after opening and then tested again. The results are shown in Table 7.

20

Table 7

Formula feed	NO release (mV/s)
Cow and Gate Premium (fresh)	0.00
Cow and Gate Premium (7 days old)	Over 1000
Milupa Aptimil First with Milupan	0.00
Sma Wysoy	0.00
Sma Gold	0.00
Farleys First	0.00
Milupa Preaptimil with Milupan	0.00

There was no activity found in any of the fresh formula milks. The Cow and Gate formula had gone off after 7 days and the NO release is probably related to bacteria (the activity was not inhibited by 100 μ M oxypurinol or DPI).

Test 8

To obtain cells used in this test, the infective bacterial strain Escherichia coli NCTC 86 (E.coli) was cultured on nutrient agar at 37° until colony formation occurred, usually overnight. This stock culture was used for subsequent experiments including sub-culturing in nutrient broth and plated onto agar weekly. Experimental cultures of E.coli were set up overnight in nutrient broth from single colonies on an agar plate. Cells were harvested and counted using a standard curve of known absorbance at 470nm against viable cell count.

Peroxynitrite was generated by the method of Crow et al, Biochem. 34, p.3544-3552 (1995). In accordance with that

method, a mixture of sodium nitrite and hydrogen peroxide was reacted under acid conditions then immediately quenched by the addition of sodium hydroxide. The concentration of ONOO^- formed was measured using an absorption coefficient of $1670 \text{ M}^{-1} \text{ cm}^{-1}$ in a spectrophotometer at a wavelength of 303nm. Solutions of different concentrations for use in (a) below were obtained by dilution of the product solution using PBS.

8(a) Peroxynitrite effect on cell viability

Cells cultured as described above were diluted to suitable working concentrations ($10^4 - 10^5 \text{ Cells Ml}^{-1}$) in sterile phosphate buffered saline (PBS). ONOO^- at a range of concentrations from $100 \mu\text{M}$ to $0.01 \mu\text{M}$ was added as a bolus dose to cells and incubated at room temperature for ten minutes. Aliquots were taken from the cell cultures and plated on to nutrient agar and incubated in a warm room at 37°C overnight. Viable cells formed colonies on the agar and were counted. The number of colonies formed was related to an untreated control and a graph of the results is shown in Figure 1, which illustrates the effect of ONOO^- on cell viability. The decrease in viable count with increasing concentration of ONOO^- in Fig. 1 is indicative of a profound inhibitory effect.

Peroxynitrite has also been shown to reduce viability in *S. enteritidis* under similar conditions to those indicated above. IC_{50} values of peroxynitrite in respect of *E. coli* and *S. enteritidis* (that is, the concentration that reduces viability to 50% of control viable count of the respective cell type) of 1.402 and $2.026 \mu\text{M}$ were determined under the conditions used.

Peroxynitrite-medicated killing has also been indicated in the case of Gram positive bacteria, in a test in which

incubation of *Staphylococcus aureus* with SIN-1 (3-morpholinosydnonimine, which releases both superoxide and NO simultaneously on hydration) showed reduced growth in dependence on the amount of added SIN-1.

5

8(b) Effect of peroxynitrite addition to bovine milk

Commercially produced bovine milk was purchased in semi-skimmed form. Aliquots were taken on day one of experimentation and plated onto nutrient agar and grown overnight at 37°C. The number of colonies formed following incubation was counted. The remaining milk was divided into three portions after removal of those aliquots. No peroxynitrite was added to the first portion, which was the control. A single bolus dose of 100µM ONOO⁻ was added to the second portion on day one, and the third portion was treated by addition of a daily bolus dose of 100µM ONOO⁻. The portions were stored during the four days of the test at a temperature of 4°C. Each day, aliquots were taken from each portion and cultured overnight at 37°C for viable count determination.

The results are shown in Fig. 2 and demonstrate that ONOO⁻ addition to milk caused a reduction in the number of colony forming units (CFU) over time compared to the control. The single bolus addition reduced the CFU significantly ($p \leq 0.01$) and the multiple dose ONOO⁻ reduced the CFU significantly below control ($p \leq 0.01$) and also significantly below the single dose ONOO⁻ ($p \leq 0.05$). Although the contaminants were not formally identified, it was postulated that they included at least *Lactobacilli* sp., that being a cell type often present in milk.

8(c) Effect of xanthine oxidase derived species on cell viability

E.coli were cultured as described above. Aliquots were taken and incubated with a reaction system consisting of

5 bovine Xanthine oxidase (XO) ($53.2 \mu\text{gml}^{-1}$), nicotinamide adenine dinucleotide in reduced form (NADH) ($300 \mu\text{M}$), sodium nitrite, (NaNO_2) 1mM and oxygen at a range of concentrations. Desired oxygen concentrations were generated by delivery into the system of a mixture of

10 oxygen and nitrogen in appropriate proportions and determined using a Clark-type O_2 electrode. This reaction was followed at 37°C for 30 min with mixing before an aliquot was taken and plated onto agar and incubated in a warm room at 37°C . Viable cells formed colonies on the

15 agar and were counted. Viable cell counts were performed in triplicate and the results expressed as a percentage viable count in each case related to a non enzyme control of the same oxygen concentration. The results are shown in Fig. 3, which suggest that XO-mediated killing of

20 cells is occurring. The most effective killing (indicated by the lowest viable cell count) is at an oxygen concentration of approximately 3% of saturation. The range of oxygen tensions used covers those in which XO has previously been considered to be active, namely

25 superoxide generation (21% O_2 saturation) and nitric oxide production (0% O_2 saturation). A certain amount of killing is seen at both of these extremes as compared with control samples. However, it is only at an intermediate oxygen concentration that the greatest

30 amount of killing is observed. This suggests a role for peroxynitrite mediated killing which has been generated in this system by the enzyme xanthine oxidase.

Corresponding data obtained in respect of *S. enteritidis* indicated a peak killing oxygen concentration of 0% for that cell type. For both *E. coli* and *S. enteritidis* the viability increases with oxygen concentration with little or no killing above about 8% oxygen, although some limited killing (about 5% and about 10% for the respective cell types) is again observed at higher oxygen concentrations of about 21%.

Replacement of NADH in the above method by $100\mu\text{M}$ hypoxanthine, with a sodium nitrite concentration of $2.5\mu\text{M}$ led to slightly higher peak killing oxygen concentrations (6.4% for *E. coli* and 1.5% for *S. enteritidis*). More limited killing was also observed using xanthine instead of hypoxanthine. The XO/hypoxanthine combination was also found to reduce the growth rate of *Lactobacillus* in a dose dependent manner for both hypoxanthine and XO. Addition to the XO/hypoxanthine system of superoxide dismutase at oxygen concentrations in the range of from 0 to 2% was found to increase the amount of measurable NO (as a result of removal of superoxide by superoxide dismutase), providing a further indication of the hypothesis that peroxynitrite formation from NO and superoxide occurs in the XO/hypoxanthine system.

8(d) Effect of peroxynitrite scavenger on bacterial growth

E. coli were seeded into nutrient broth at $2 \cdot 10^7$ cell ml^{-1} and incubated at 37°C under atmospheric air conditions (that is, 21% oxygen saturation). Four separate test samples of volume 1 ml were prepared by addition at 1 hour of incubation as follows:

- 1 Peroxynitrite at concentration $100\mu\text{M}$.

- 2 Peroxynitrite at 100 μ M and quercetin (peroxynitrite scavenger) at 100 μ M.
- 3 Xanthine oxidase (53.2 μ g) and xanthine at 100 μ M.
- 4 Xanthine oxidase (53.2 μ g) and NADH at 100 μ M.

5

Corresponding controls were also prepared, and the growth curves generated over time determined by monitoring absorbance. Sample (1) showed strong killing, but the presence of quercetin (sample (2)) had a clear effect in
10 reducing killing, pointing towards peroxynitrite mediation of killing. XO/NADH (sample (4)) and XO/xanthine (sample (3)) showed limited, but significant, growth retardation effects.

15

The results of parts (a), (b) and (d) of this test appear to confirm the bactericidal potency of the peroxynitrite species. Part (c) above supports the hypothesis that, under appropriate conditions, XOR can catalyse production both of superoxide and of NO, interaction of those two products giving rise to peroxynitrite, and possibly other
20 interaction products that may have similar bactericidal activity to peroxynitrite. The oxygen concentrations of under 8% at which maximum killing was observed is believed to be similar to that in the neonatal gut.

25

8(e) Effect of hypoxanthine addition on bacterial growth in pasteurised milk.

30

Pasteurised semi skimmed milk 1 ml was aliquoted into 7 sterile plastic bijou bottles comprising control and treated hypoxanthine (Hxan) groups. To the control group at day 0 was added 10 μ l of sterile ddH₂O and to the treated group 10 μ l of hypoxanthine solution was added to give a final concentration of 100 μ M. The samples were stored refrigerated at 4-8°C until required on a specific

day when a control and treated sample was incubated at 37°C for 30 minutes. A 100µl aliquot was taken from each sample and spread onto nutrient agar which was then incubated over night at 37°C. The total number of colonies formed regardless of type was counted and expressed for each treatment as the colony forming units per ml of the original test sample (CFU ml⁻¹). The results are shown in Fig. 4.

The control group showed significant colony formation from day 1. The effect of the addition of hypoxanthine was to reduce the total number of colonies formed for the length of the experiment (8 days).

8(f) Effect of XO and hypoxanthine incubation on bacterial growth rate.

Bacteria were seeded into nutrient broth (NB) and grown over night at 37°C. The culture was then counted and normalised to give a final cell concentration of 1.8×10^7 cells well⁻¹ into the wells of a 96 well plate in fresh nutrient broth. The optical density of each well was monitored every 15 minutes as a measure of the growth rate of each bacterial species. Growth curves were generated for each species and the maximal rate of growth (logarithmic phase) was measured. Cells were treated to a range of experimental conditions in which the growth rate of cells was related to a control of untreated cells. The results were expressed as a percentage of the growth rate of the control cells.

- (i) At a fixed concentration of hypoxanthine (100µM) a range of purified XO protein concentrations were added at time 0 minutes. The optical density was followed and the growth rate calculated. The effect of enzyme concentration is shown in Fig. 5

for *Staphylococcus aureus* 6751 and *Lactobacillus casei* 6375.

The growth rate of both *Staphylococcus* and *Lactobacilli* was reduced compared to the control. *Lactobacillus* growth rate was reduced with a half maximal concentration of XO (that is, the concentration at which the cell growth was 50% of control) being about 14µg. A less marked growth inhibition was observed for *Staphylococcus*.

(ii) The growth rate was also measured in relation to the concentration of hypoxanthine, using 30µg of XO with cells seeded as described above. Fig. 6 shows the effect of hypoxanthine concentration on bacterial growth. Hypoxanthine in the presence of XO reduced the growth rate of both bacterial species with greatest effect on the *Lactobacillus*. The half maximal hypoxanthine dose at 30µg XO was 103.5µM.

(iii) To show that the effect of XO and Hypoxanthine addition was due to the enzymically derived products, oxypurinol at a range of concentrations was added to the cells in the presence of 30µg XO and 200µM hypoxanthine. Fig. 7 shows its effect on the growth rate of *Lactobacillus*.

Oxypurinol had no effect on growth rate when added at the highest concentration when added alone. However the effect of oxypurinol was to reduce the effect of XO/hypoxanthine growth inhibition in a dose dependent manner.

(iv) The effect of XO hypoxanthine addition was measured on the growth rate of a range of bacterial species with 30µg XO at varying

hypoxanthine concentrations using cells seeded as described above. The results of this study are shown in Fig. 8.

8(g) Effect of H₂O₂ and peroxyxynitrite on the growth rate of bacteria

To determine the effect of possible enzymically generated radical species cells were grown in the presence of hydrogen peroxide or peroxyxynitrite. Cells of various species were grown as previously described and a bolus addition of either H₂O₂ or peroxyxynitrite was added at the beginning of logarithmic growth, previous experiments having showed this treatment as the most effective. The cell growth was determined and plotted against the concentration of H₂O₂ or peroxyxynitrite, as appropriate, and the half maximal concentration of H₂O₂ or peroxyxynitrite was determined. The results are summarised in Table 8, in which corresponding half maximal concentrations for the XO/hypoxanthine system are also given.

Table 8

Species	Half maximal concentration		
	XO/Hxan (μM Hypoxanthine)	H ₂ O ₂ (μM)	ONOO - (μM)
<i>Bacillus sp</i>	39.8	77.6	
<i>Micrococcus sp</i>	25.1	19.5	
<i>Lactobacillus casei</i>	103.5	304.8	55
<i>Staphylococcus aureus</i>		—	290
<i>E. coli</i>			1.4
<i>Salmonella enteritidis</i>			2.0

As shown in Table 8, the half maximal concentrations for peroxynitrite in respect of *Lactobacillus casei* is much lower than for H_2O_2 . In respect of *S. aureus*, the growth inhibition in the presence of H_2O_2 was very limited, with
5 50% growth reduction not being observed at the concentration ranges used.

The following Example illustrates the invention:

10 **Example 1**

The effect of the addition of XOR to formula feed on the generation of NO was investigated. Different compositions comprising one or more of buttermilk, formula feed, XOR, nitrite and NADH were studied under
15 hypoxic conditions.

The samples were tested for NO generation using the method described above.

To the "milk" bijou were added either 598µl Cow and Gate formula feed and 2µl XOR or 100µl buttermilk and
20 500µl 1X PBS to give a total volume in the "milk" bijou of 600µl in each case.

Table 9

Sample	20mM nitrite added?	1mM NADH added?	NO release (mV/s)
Formula feed and XOR	Yes	Yes	247.3
Formula feed and XOR	No	No	0.0
Formula feed and XOR	Yes	No	0.0
Formula feed and XOR	No	Yes	0.0
Formula feed and 1X PBS	Yes	Yes	0.0
Buttermilk	Yes	Yes	379.1

Table 9 shows that where no XOR is added to the formula feed, there is no generation of NO. That is consistent with the applicant's findings that formula feeds do not contain active XOR.

5 The addition of XOR to the formula feed in the presence of nitrite and NADH led to the generation of NO. The omission of either the nitrite or the NADH gave no NO generation. As indicated above, it is believed that both a source of nitrite and an electron donor substrate will
10 be available *in vivo* in the GI tract and that in many cases specific further addition of nitrite and/or NADH (or other substrate) will not be required.

 Buttermilk alone, without the addition of any of XOR, nitrite or NADH, led to the generation of NO. As
15 indicated above, buttermilk contains active XOR as well as a source of nitrite, and an electron donor substrate.

Claims:

1. A formula feed for administration to a human or animal, the formulation including active xanthine oxidoreductase (XOR).
5
2. A formula feed according to claim 1, which includes at least 50 μ g/ml active xanthine oxidoreductase.
3. A formula feed according to claim 1 or claim 2, in which the concentration of XOR exceeds the normal
10 physiological concentration of XOR.
4. A formula feed according to any one of claims 1 to 3, which includes up to 500 μ g/ml active xanthine oxidoreductase.
5. A formula feed according to claim 4, in which the
15 formulation includes from 50 to 150 μ g/ml of XOR.
6. A formula feed according to any one of claims 1 to 5, in which the formulation includes buttermilk, the buttermilk including active XOR.
7. A formula feed according to any one of claims 1
20 to 6, in which the formulation is in liquid form.
8. A formula feed according to any one of claims 1 to 7, the formulation further including one or more electron donors.
9. A formula feed according to any one of claims 1
25 to 8, the formulation further including one or more electron acceptors.
10. A formula feed according to any one of claims 1 to 9, which is suitable for feeding a human infant.
11. A combination product for use in the preparation
30 of a formula feed according to any one of claims 1 to 10, in which the product comprises two separate portions, the first portion including active XOR and the second portion

- 35 -

being sterilised and comprising substantially no active XOR.

12. A combination product according to claim 11, in which the second portion is in the form of a powder.

5 13. A combination product according to claim 11 or claim 12, in which the second portion has been heat treated.

14. A combination product according to any one of claims 11 to 13, in which the first portion has been
10 pasteurised.

15. A combination product according to any one of claims 11 to 14, in which the first portion is in a first container, and the second portion is in a second container.

16. A composition for use in the treatment of
15 gastrointestinal infection, the composition being suitable for addition to a formula feed, and the composition comprising active XOR in combination with one or more electron donors and/or one or more electron acceptors.

17. A composition according to claim 16, the
20 composition comprising buttermilk.

18. A composition according to claim 16 or claim 17, the composition being in the form of a powder.

19. A method of making a formula feed, the method comprising the step of adding a composition comprising
25 active XOR.

20. A method according to claim 19, the composition being according to any one of claims 16 to 18.

21. A method according to claim 19 or claim 20, the method comprising the steps of:

30 a. preparing a first portion of the formula feed, comprising a composition including active XOR; and

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b. preparing a second portion of the formula feed, the first portion and second portion being separate from each other for subsequent mixing to form the formula feed.

22. A method according to claim 21, in which the
5 first portion comprises lyophilised buttermilk.

23. A method according to claim 21, in which the first portion comprises buttermilk that has been spray-dried at a temperature which is so selected that the activity of the XOR is at least substantially retained.

10 24. A method according to any one of claims 21 to 23, in which the first portion has been subjected to a heat treatment step in which its temperature does not exceed 65°C.

25. A method according to claim 21 or claim 22, in
15 which the second portion comprises a sterilised feed composition.

26. A formulation for use in the treatment of gastrointestinal infection, the formulation being suitable for administration as a feed and including active xanthine
20 oxidoreductase.

27. Use of active XOR in the treatment of gastrointestinal (GI) infection.

28. Use of active XOR in the treatment of Scours disease.

25 29. Use of active XOR in the killing of bacteria.

30. Method of feeding a patient with enteral feed, in which the enteral feed includes active XOR.

31. Method of feeding an infant with formula feed, in which the formula feed includes active XOR.

30 32. A formulation comprising XOR substantially as described in Example 1 herein.

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33. A formulation for use as a bactericidal agent in the human or animal digestive system, the formulation including active xanthine oxidoreductase (XOR).

34. A feed formulation according to claim 33, which
5 is suitable for use as an enteral feed.

35. A feed formulation according to claim 33, which is for use in the treatment of Scours disease.

10

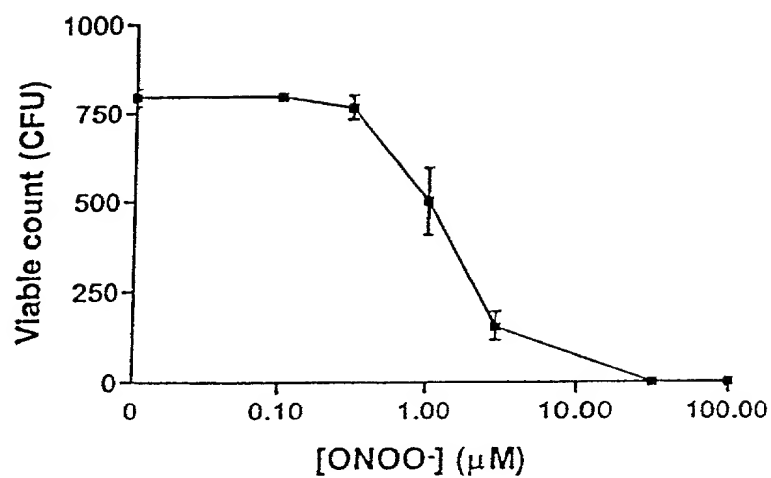


Fig. 1

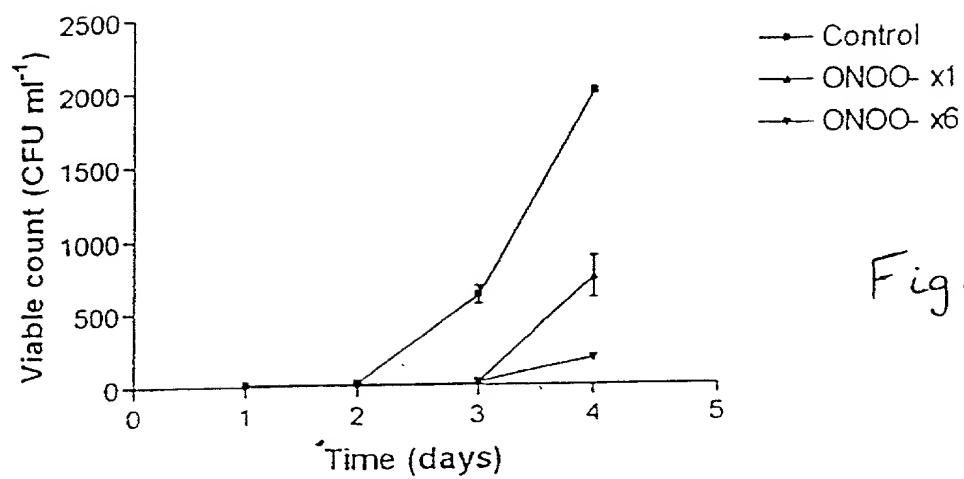


Fig. 2

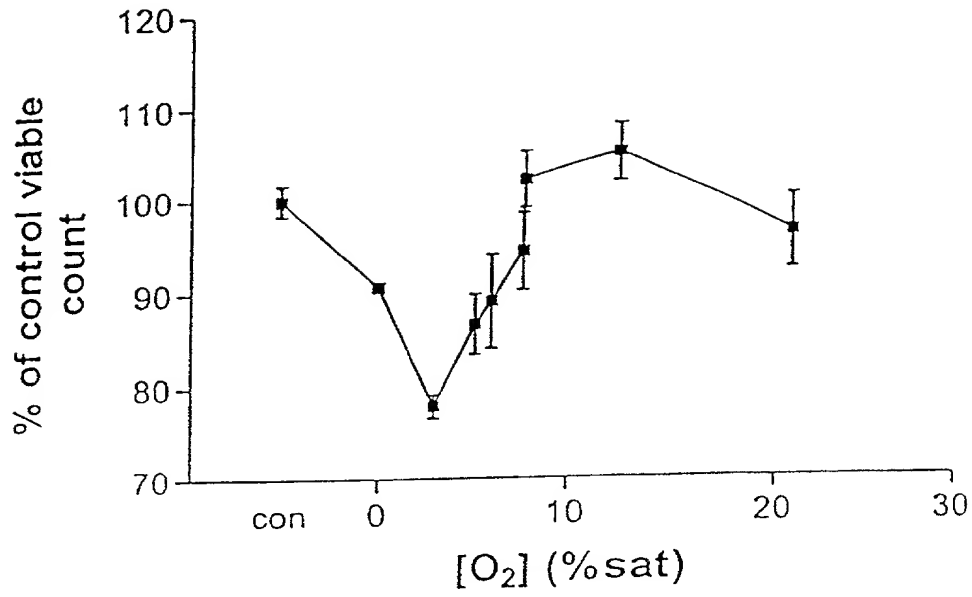


Fig.3

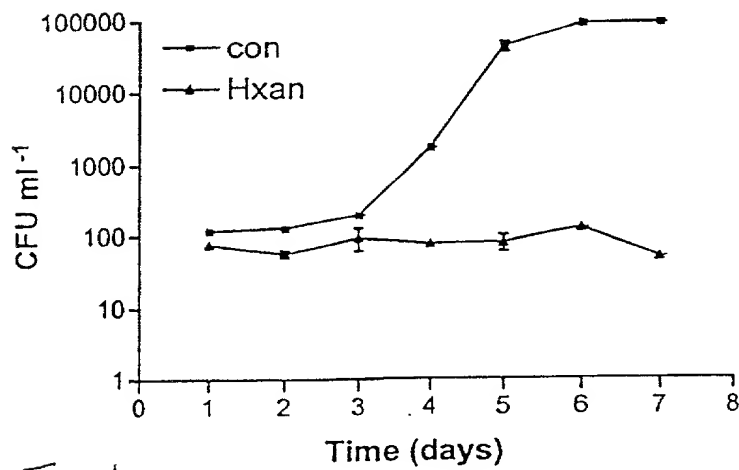


Fig.4

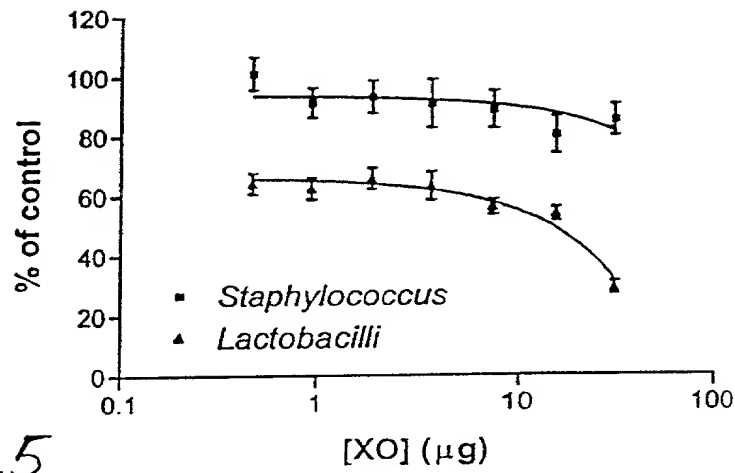


Fig. 5

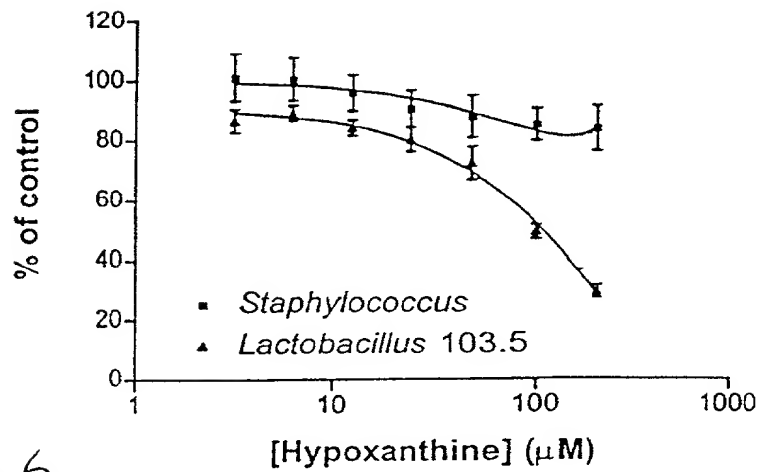


Fig. 6

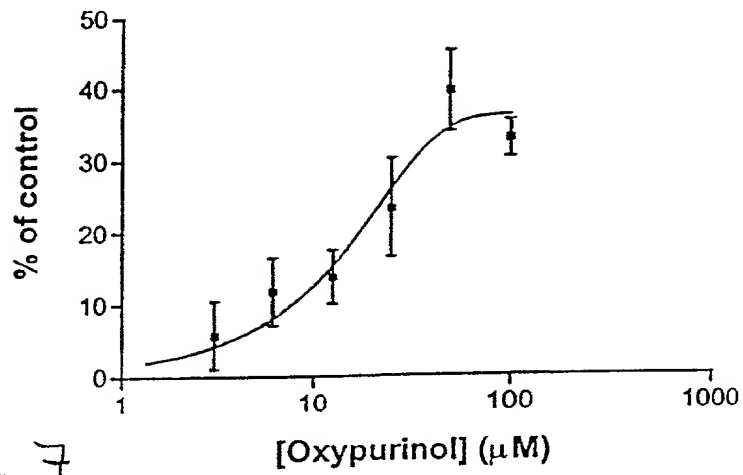


Fig. 7

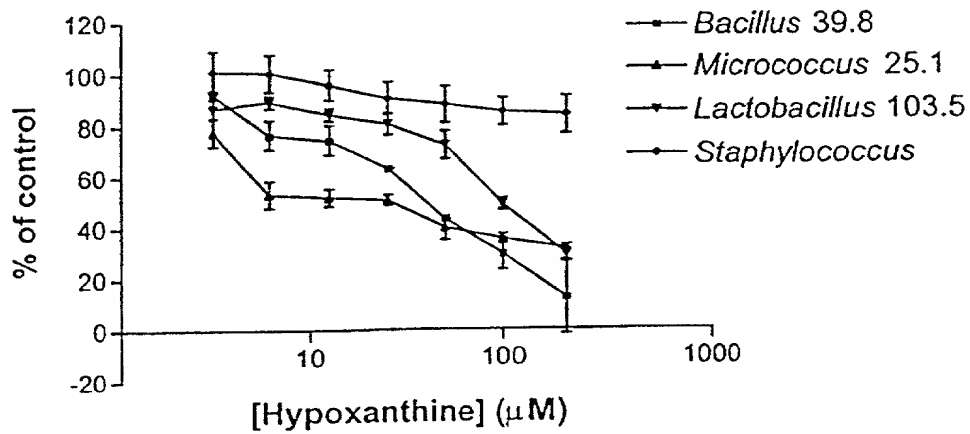


Fig. 8

MERCHANT & GOULD P.C.

United States Patent Application

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor I hereby declare that my residence, post office address and citizenship are as stated below next to my name; that

I verify believe I am the original, first and sole inventor (if only one name is listed below) or a joint inventor (if plural inventors are named below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: **INGESTIBLE COMPOSITIONS COMPRISING ANTIBACTERIAL AGENTS**

The specification of which

- a. ☐ is attached hereto
b. ☒ was filed on as application serial no. and was amended on (if applicable) (in the case of a PCT-filed application) described and claimed in international no. PCT/GB99/02845 filed August 27, 1999 and as amended on December 4, 2000 (if any), which I have reviewed and for which I solicit a United States patent.

I hereby state that I have reviewed and understood the contents of the above-identified specification, including the claims, as amended by any amendments referred to above.

I hereby claim foreign priority benefits under Title 35, United States Code, § 119/365 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on the basis of which priority is claimed:

- a. ☐ no such applications have been filed.
b. ☒ such applications have been filed as follows:

FOREIGN APPLICATION(S), IF ANY, CLAIMING PRIORITY UNDER 35 USC § 119			
COUNTRY	APPLICATION NUMBER	DATE OF FILING (day, month, year)	DATE OF ISSUE (day, month, year)
Great Britain	9218913.7	28 August 1996	
Great Britain	9227340.8	10 December 1996	
ALL FOREIGN APPLICATION(S), IF ANY, FILED BEFORE THE PRIORITY APPLICATION(S)			
COUNTRY	APPLICATION NUMBER	DATE OF FILING (day, month, year)	DATE OF ISSUE (day, month, year)

I hereby claim the benefit under Title 35, United States Code, § 120/365 of any United States and PCT international application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. APPLICATION NUMBER	DATE OF FILING (day, month, year)	STATUS (pending, pending, abandoned)

I hereby claim the benefit under Title 35, United States Code § 119(c) of any United States provisional application(s) listed below:

U.S. PROVISIONAL APPLICATION NUMBER	DATE OF FILING (Day, Month, Year)

I acknowledge the duty to disclose information that is material to the patentability of this application in accordance with Title 37, Code of Federal Regulations, § 1.56 (reprinted below):

§ 1.56 Duty to disclose information material to patentability.

(a) A patent by its very nature is affected with a public interest. The public interest is best served, and the most effective patent examination occurs when, at the time an application is being examined, the Office is aware of and evaluates the teachings of all information material to patentability. Each individual associated with the filing and prosecution of a patent application has a duty of candor and good faith in dealing with the Office, which includes a duty to disclose to the Office all information known to that individual to be material to patentability as defined in this section. The duty to disclose information exists with respect to each pending claim until the claim is canceled or withdrawn from consideration, or the application becomes abandoned. Information material to the patentability of a claim that is canceled or withdrawn from consideration need not be submitted if the information is not material to the patentability of any claim remaining under consideration in the application. There is no duty to submit information which is not material to the patentability of any existing claim. The duty to disclose all information known to be material to patentability is deemed to be satisfied if all information known to be material to patentability of any claim issued in a patent was cited by the Office or submitted to the Office in the manner prescribed by §§ 1.97(b)-(d) and 1.98. However, no patent will be granted on an application in connection with which fraud on the Office was practiced or attempted or the duty of disclosure was violated through bad faith or intentional misconduct. The Office encourages applicants to carefully examine:

- (1) prior art cited in search reports of a foreign patent office in a counterpart application, and
 - (2) the closest information over which individuals associated with the filing or prosecution of a patent application believe any pending claim patentably defines, to make sure that any material information contained therein is disclosed to the Office.
- (b) Under this section, information is material to patentability when it is not cumulative to information already of record or being made of record in the application, and
- (1) It establishes, by itself or in combination with other information, a *prima facie* case of unpatentability of a claim;
 - (2) It refutes, or is inconsistent with, a position the applicant takes in:
 - (i) Opposing an argument of unpatentability relied on by the Office, or
 - (ii) Asserting an argument of patentability.

A *prima facie* case of unpatentability is established when the information compels a conclusion that a claim is unpatentable under the preponderance of evidence, burden-of-proof standard, giving each term in the claim its broadest reasonable construction consistent with the specification, and before any consideration is given to evidence which may be submitted in an attempt to establish a contrary conclusion of patentability.

- (c) Individuals associated with the filing or prosecution of a patent application within the meaning of this section are:
- (1) Each inventor named in the application;
 - (2) Each attorney or agent who prepares or prosecutes the application; and
 - (3) Every other person who is substantively involved in the preparation or prosecution of the application and who is associated with the inventor, with the assignee or with anyone to whom there is an obligation to assign the application.
- (d) Individuals other than the attorney, agent or inventor may comply with this section by disclosing information to the attorney, agent, or inventor.
- (e) In any continuation-in-part application, the duty under this section includes the duty to disclose to the Office all information known to the person to be material to patentability, as defined in paragraph (b) of this section, which became available between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application.

I hereby appoint the following attorney(s) and/or patent agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected herewith:

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Bemis, John M.	Reg. No. 43,496	McDonald, Daniel W.	Reg. No. 32,044
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Gould, John D.	Reg. No. 18,223	Summer, John P.	Reg. No. 29,114
Gregson, Richard	Reg. No. 41,804	Swenson, Erik G.	Reg. No. 45,147
Gressens, John J.	Reg. No. 33,112	Tellekson, David K.	Reg. No. 32,314
Hamer, Samuel A.	Reg. No. 46,754	Trembath, Jon R.	Reg. No. 38,344
Hamer, Curtis B.	Reg. No. 29,165	Tuchman, Ida	Reg. No. 45,924
Harrison, Kevin C.	Reg. No. 46,759	Tunheim, Marcia A.	Reg. No. 42,189
Hertzberg, Brett A.	Reg. No. 42,660	Underhill, Albert L.	Reg. No. 27,403
Hillson, Randall A.	Reg. No. 31,838	Vandenburg, J. Derek	Reg. No. 32,179
Holzer, Jr., Richard J.	Reg. No. 42,668	Wahl, John R.	Reg. No. 33,044
Johnston, Scott W.	Reg. No. 39,721	Werner, Karrie G.	Reg. No. 43,245
Kadlovich, Namilo D.	Reg. No. 34,196	Welter, Paul A.	Reg. No. 20,890
Karjaker, Shaulat	Reg. No. 34,049	Whipps, Brian	Reg. No. 43,261
Katzelberger, Denise	Reg. No. 33,924	Whitaker, John E.	Reg. No. 42,222
Kays, Jeramie J.	Reg. No. 42,724	Wickham, J. Scott	Reg. No. 41,376
Knecht, Homer L.	Reg. No. 21,197	Williams, Douglas J.	Reg. No. 27,054
Kowalczyk, Alan W.	Reg. No. 31,535	Withers, James D.	Reg. No. 40,376
Kowalczyk, Katherine M.	Reg. No. 36,848	Wiz, Joanne	Reg. No. 41,980
Lacy, Paul E.	Reg. No. 38,946	Wu, Tong	Reg. No. 43,361
Larson, James A.	Reg. No. 40,443	Xu, Min S.	Reg. No. 39,536
Leon, Andrew J.	Reg. No. 46,869	Zerli, Anthony R.	Reg. No. 45,255

I hereby authorize them to act and rely on instructions from and communicate directly with the person/assignee/attorney/firm/organization who/which first sends/sent this case to them and by whom/which I hereby declare that I have consented after full disclosure to be represented unless/until I instruct Merchant & Gould P.C. to the contrary.

Please direct all correspondence in this case to Merchant & Gould P.C. at the address indicated below:

Merchant & Gould P.C.
P.O. Box 2903
Minneapolis, MN 55402-0903



I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

2	Full Name of Inventor	Family Name	State	First Given Name	David	Second Given Name	Russell
4	Residence & Citizenship	City	Bath	State or Foreign Country	Great Britain	Country of Citizenship	Great Britain
1	Mailing Address	Address		City	Bath	State & Zip Code/Country	BA1 2ET / Great Britain
				The Ground Floor Flat 16 The Circus			
Signature of Inventor 201:						Date: 19/03/01	

2	Full Name of Inventor	Family Name	Sevens	First Given Name	Clifford	Second Given Name	Robert
4	Residence & Citizenship	City	Wiltshire	State or Foreign Country	Great Britain	Country of Citizenship	Great Britain
2	Mailing Address	Address		City	Wiltshire	State & Zip Code/Country	BA15 1TL / Great Britain
				49 The Old Rectory, Bradford on Avon			
Signature of Inventor 202:						Date: 19/3/01	

2	Full Name of Inventor	Family Name	Elschall	First Given Name	Robert	Second Given Name	
4	Residence & Citizenship	City	Bath	State or Foreign Country	Great Britain	Country of Citizenship	Great Britain
2	Mailing Address	Address		City	Bath	State & Zip Code/Country	BA1 4LR / Great Britain
				20 Lansdown Lane			
Signature of Inventor 203:						Date: 27.03.01	

2	Full Name of Inventor	Family Name	Harrison	First Given Name	Roger	Second Given Name	
4	Residence & Citizenship	City	Wiltshire	State or Foreign Country	Great Britain	Country of Citizenship	Great Britain
4	Mailing Address	Address		City	Wiltshire	State & Zip Code/Country	BA15 1EH / Great Britain
				16 Grove Lane, Bradford on Avon			
Signature of Inventor 204:						Date: 19/03/01	

2	Full Name of Inventor	Family Name	Muller	First Given Name	Timothy	Second Given Name	None MARC
4	Residence & Citizenship	City	Bath	State or Foreign Country	Great Britain	Country of Citizenship	Great Britain
5	Mailing Address	Address		City	Bath	State & Zip Code/Country	BA1 1LT / Great Britain
				7-9 UNIVERSITY OF 110 Langford Road, Southdown		692 74Y	
Signature of Inventor 205:						Date: 21/3/01	

2	Full Name Of Inventor	Family Name Edwards <i>John Edwards</i>	First Given Name Richard	Second Given Name
4	Residence & Citizenship	City Wolverhampton	State or Foreign Country Great Britain	Country of Citizenship Great Britain
6	Mailing Address	Address 2 Southville Road, Bradford on Avon	City Wolverhampton	State & Zip Code/Country BA15 1HP / Great Britain
Signature of Inventor 286: <i>John Edwards</i>			Date 24.07.01	
1	Full Name Of Inventor	Family Name	First Given Name	Second Given Name
3	Residence & Citizenship	City	State or Foreign Country	Country of Citizenship
5	Mailing Address	Address	City	State & Zip Code/Country
Signature of Inventor 287:			Date	

United States Patent Application

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor I hereby declare that: my residence, post office address and citizenship are as stated below next to my name; that

I verily believe I am the original, first and sole inventor (if only one name is listed below) or a joint inventor (if plural inventors are named below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: INGESTIBLE COMPOSITIONS COMPRISING ANTIBACTERIAL AGENTS

The specification of which

- a. ☐ is attached hereto
- b. ☒ was filed on as application serial no. and was amended on (if applicable) (in the case of a PCT-filed application) described and claimed in international no. PCT/GB99/02845 filed August 27, 1999 and as amended on December 4, 2000 (if any), which I have reviewed and for which I solicit a United States patent.

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FOREIGN APPLICATION(S), IF ANY, CLAIMING PRIORITY UNDER 35 USC § 119			
COUNTRY	APPLICATION NUMBER	DATE OF FILING (day, month, year)	DATE OF ISSUE (day, month, year)
Great Britain	9818913.7	28 August 1998	
Great Britain	9827243.8	10 December 1998	
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U.S. APPLICATION NUMBER	DATE OF FILING (day, month, year)	STATUS (patented, pending, abandoned)

I hereby claim the benefit under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below:

U.S. PROVISIONAL APPLICATION NUMBER	DATE OF FILING (Day, Month, Year)

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(3) Every other person who is substantively involved in the preparation or prosecution of the application and who is associated with the inventor, with the assignee or with anyone to whom there is an obligation to assign the application.

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I hereby appoint the following attorney(s) and/or patent agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected herewith:

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Hamer, Curtis B.	Reg. No. 29,165	Tuchman, Ido	Reg. No. 45,924
Harrison, Kevin C.	Reg. No. 46,759	Tunheim, Marcia A.	Reg. No. 42,189
Hertzberg, Brett A.	Reg. No. 42,660	Underhill, Albert L.	Reg. No. 27,403
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Kowalchyk, Katherine M.	Reg. No. 36,848	Witt, Jonelle	Reg. No. 41,980
Lacy, Paul E.	Reg. No. 38,946	Wu, Tong	Reg. No. 43,361
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Leon, Andrew J.	Reg. No. 46,869	Zeuli, Anthony R.	Reg. No. 45,255

I hereby authorize them to act and rely on instructions from and communicate directly with the person/assignee/attorney/firm/ organization who/which first sends/sent this case to them and by whom/which I hereby declare that I have consented after full disclosure to be represented unless/until I instruct Merchant & Gould P.C. to the contrary.

Please direct all correspondence in this case to Merchant & Gould P.C. at the address indicated below:

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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